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Changes in intracellular levels of $A_{p3}A$ and $A_{p4}A$ in cysts and larvae of *Artemia* do not correlate with changes in protein synthesis after heat-shock

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ABSTRACT

Artemia larvae respond to a brief heat-shock between 28° and 40°C with an increase in the synthesis of two groups of proteins of M_r 68,000 and 89,000. At 40°C synthesis of all other proteins is strongly repressed. Cysts, which are naturally thermotolerant, synthesise both heat-shock proteins at temperatures up to 47°C but maintain normal protein synthesis. During pre-emergence development, Ap_3A is present in cysts at a concentration twice that of Ap_4A. The maximum level of 7.6pmol/10^6 cells is reached shortly before hatching of the larvae. After hatching, the levels of both nucleotides decline. A 40°C heat-shock produces a 1.8-fold increase in both nucleotides within 20 min in cysts and larvae. A 2.8-fold increase results from a 47°C heat-shock to cysts. The rates of increase parallel but do not precede the increases in the heat-shock proteins. Since non-heat-shocked cysts possess higher levels of Ap_3A and Ap_4A than do heat-shocked larvae, the observed heat-induced changes in gene expression cannot be explained simply in terms of the intracellular concentrations of these nucleotides.

INTRODUCTION

When exposed to temperatures significantly above their normal growth temperature, all cells and organisms studied so far respond with an enhanced or de novo transcription and preferential translation of specific restricted sets of proteins, the so-called heat-shock proteins (hsp's) [1,2]. Many of these hsp's are also induced by a wide variety of chemical agents including ethanol, arsenite and cadmium. Regulation of the control of hsp synthesis occurs both at the transcriptional and translational level; a specific heat-shock transcription factor has been detected in Drosophila [3] while heat-induced modifications to translational initiation factors have also been reported [4,5].

Despite the wealth of information available on the hsp's and their genes, the nature of the transducing signals which mediate between the various stresses and the subsequent changes in RNA and protein synthesis remains unclear. One suggestion is that the common factor among these inducing stresses is their ability to denature proteins, leading to an overloading of a ubiquitin-dependent system of intracellular proteolysis. Hypo-ubiquitinylation of heat-shock transcription factors

may then promote their binding to the regulatory elements of the hsp genes [6]. Another property common to many inducing agents is the production of oxidative damage. In Escherichia coli and Salmonella typhimurium an early response to exposure to oxidising agents and to heat-shock is the accumulation of a set of unusual nucleotides, the bis (5'-nucleosidyl) oligophosphates [7-9]. For example, on shifting from 28° to 50°C these nucleotides rapidly rose from undetectable levels to 30μM for bis (5'-adenosyl) tetraphosphate (Ap_4A), 40μM for bis (5'-adenosyl) triphosphate (Ap_3A), 32μM for adenosine 5'-P^1-tetraphospho-P^4-5"-guanosine (Ap_4G) and 18μM for adenosine-5'-P^1-triphospho-P^3-5'"-guanosine (Ap_3G) [8]. A milder heat-shock from 28° to 42°C resulted in approximately 4-fold lower increases in Ap_4A, Ap_3A, and Ap_3G with no increase in Ap_4G [8] while treatment with cadmium chloride caused dramatic increases to between 138 and 365μM in all but Ap_4G [9]. Since an accumulation of Ap_3A has also been reported in heat-shocked yeast cells [10] it is possible that these nucleotides act as alarmones, signalling the onset of metabolic stress and regulating the stress response [11]. They are synthesised in vitro, and presumably in vivo, by the substitution of pyrophosphate by a nucleotide in the back reaction of aminoacyl-AMP formation catalysed by certain aminoacyl-tRNA synthetases [12,13].

In addition to its postulated role as an alarmone, Ap_4A has also been implicated in the initiation of DNA replication as it appears to promote premature DNA synthesis in quiescent cells [14,15] and binds to and primes the replicative DNA polymerase-α holoenzyme in vitro [16,17]. We have previously demonstrated a 125-fold increase in the intracellular level of Ap_4A during pre-emergence development of embryonic cysts of the brine shrimp Artemia. The maximal level of 3.8 pmol/10^6 cells (3.3μM) coincides with hatching and the onset of DNA replication [18]. Such a correlation has also been noted with mammalian cells [19] and sea urchin embryos [20]. As Artemia cysts are intrinsically more tolerant to stress than newly hatched larvae, they provide a useful system for examining the possible additional role of Ap_4A and related nucleotides in the stress response. Here we report on the effect of heat-shock on protein synthesis and on the intracellular levels of Ap_3A and Ap_4A in Artemia cysts and larvae.

MATERIALS AND METHODS

Great Salt Lake Artemia cysts were prepared, decapsulated and hatched as described previously [18,21]. [^{3}H]Ap_4A (15Ci/mmol) and $NaH^{14}CO_3$ (50mCi/mmol) were from Amersham. [^{3}H]Ap_3A was the generous gift of Dr. A. Ogilvie, Erlangen, GFR.

Protein labelling and analysis

Heat-shocks were delivered by immersion of aliquots of cysts or larvae contained in 3ml polystyrene tubes in a water bath at the appropriate temperature. After heat-

shock, 25mg wet wt. samples of cysts or larvae were collected by filtration on 2.5 cm paper discs and transferred to 1ml of thoroughly degassed seawater containing 50µCi NaH $^{14}CO_3$ in a 3ml polystyrene tube. The tubes were flushed with O_2, sealed and incubated at 28°C for 3h. Organisms were harvested and washed with ice-cold 20mM potassium phosphate buffer pH 7.2, 1mM EDTA. Samples were then homogenised in a Kontes glass homogeniser in 250µl 10mM potassium phosphate pH 7.2, 0.3M KCl, 1mM 2-mercaptoethanol, 1mM EDTA, 10mM $NaHSO_3$, 2.5mM phenylmethylsulphonyl fluoride, 50 µg/ml soybean trypsin inhibitor. The homogenates were centrifuged (16,000g, 10 min, 4°C) and the supernatants retained and dialysed extensively against 62.5mM Tris-HCl pH 6.8, 1mM EDTA. Protein concentrations were determined by the dye-binding method [22].

Samples corresponding to the same numbers of organisms in each case (50µg protein; 1500-7000 cpm) were subjected to electrophoresis in 10% SDS-polyacrylamide gels [23]. Incorporation of the ^{14}C label was visualised by impregnating the gel with Amplify fluorographic reagent (Amersham) and exposing the dried gel to a pre-flashed sheet of Fuji RX film at -70°C for between 4 days and 3 weeks, depending on the degree of incorporation of label [24]. Fluorographs were scanned in a Beckman DU-8B spectrophotometer.

Nucleotide analysis

1g portions of cysts or larvae in 100ml seawater were brought rapidly to the appropriate heat-shock temperature by swirling in a 60°C water bath followed by transfer to the required incubation temperature. TCA extracts were prepared and analysed by reversed phase ion-pair chromatography as previously described [18]. Replacement of the Radial-Pak C_{18} column by an RP-18 column yielded base-line resolution of Ap_3A and Ap_4A with the same solvent system as used previously. Typical retention volumes were AMP, 8.4ml; ADP, 11.3ml; ATP, 13.1ml; Ap_3A 29.7ml and Ap_4A, 34.7ml, hence [^3H]Ap_3A and [^3H]Ap_4A could be used unambiguously as tracers when added to the homogenates. The endogenous content of Ap_3A and Ap_4A in pooled fractions was determined using the coupled bioluminescence assays described previously [18,25]. Confirmation of the identity of the endogenous nucleotides was carried out as before [18].

RESULTS

Effect of heat-shock on protein synthesis in cysts and larvae

The period of pre-emergence development in Artemia lasts about 14h from the time the desiccated gastrular cyst is rehydrated until the differentiated pre-nauplius larva emerges from the cuticle. It is characterised by rapid protein and RNA synthesis but an absence of DNA synthesis. Replication recommences only upon emergence [18,26]. During this time cysts are considerably more thermotolerant than newly hatched larvae, being able to withstand temperatures up to 49°C, while larvae are killed at 42°C (Miller and McLennan, unpublished data). Full details of the heat-shock response in Artemia will be published elsewhere.

The thermotolerance of cysts is reflected in the pattern of protein synthesis observed after heat-shock. Newly hatched larvae respond to a mild 1h heat shock at 32°C with an increase in the synthesis of a polypeptide of M_r 89,000 (hsp 89) (Fig. 1A). As the heat-shock temperature is increased, the preferential synthesis of a second polypeptide of M_r 68,000 (hsp 68) is apparent until at 39°C these two are the major translational products. A further increase in the heat-shock temperature of

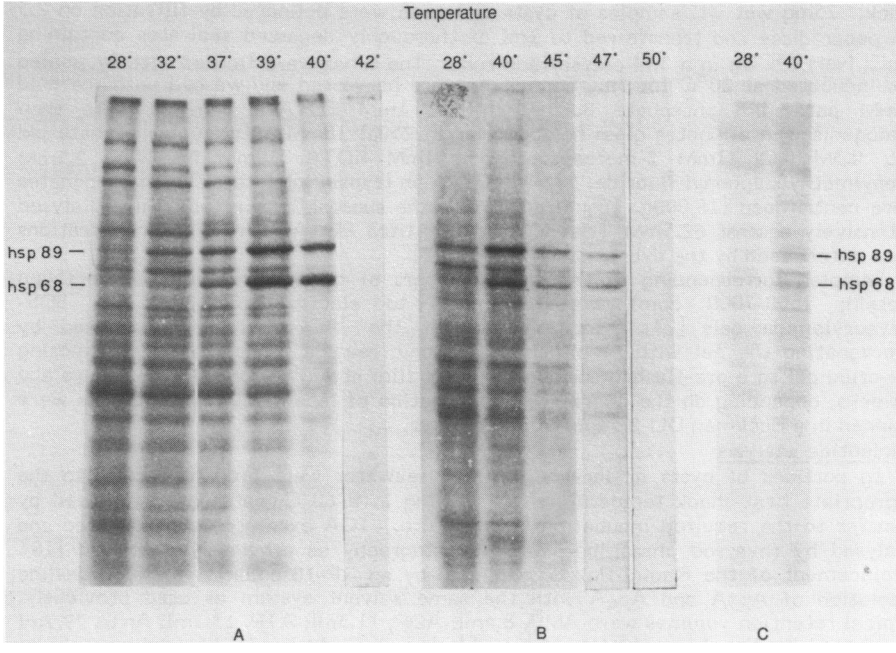

Fig. 1: Effect of heat-shock at different temperatures on protein synthesis in larvae and cysts (A) 24h larvae, (B) 2h cysts and (C) 12h cysts were incubated at the temperatures indicated for 1h before returning to 28°C, labelling with $NaH^{14}CO_3$ and analysing as described in Materials and Methods.

one degree virtually eliminates the synthesis of all other proteins. This response is identical in larvae at all stages up to 96h. In these respects Artemia larvae behave like many other systems which have been studied [1,2].

Cysts which have been redeveloping for 2h also respond to heat-shock with an increase in the synthesis of hsp 68 and hsp 89, however non-hsp synthesis is not preferentially inhibited, even at 47°C (Fig. 1B). This naturally thermotolerant state of the cysts is similar to that which can be induced in other systems by the application of a previous, sub-lethal heat-shock [27] and is preserved up to the point of emergence. 12h cysts also maintain the normal pattern of protein synthesis at 40°C in addition to increasing the synthesis of hsp 68 and hsp 89 (Fig. 1C).

The rates of appearance of hsp 68 and hsp 89 during a heat-shock to larvae at 40°C have also been examined. A 5-minute shock is sufficient to cause a significant increase in the synthesis of both proteins, particularly hsp 89 (Fig. 2.). A heat-shock of between 15 and 30min duration results in maximum rates of synthesis of both species while causing a 35% reduction in the synthesis of non-hsp's. A reduction in the synthesis of hsp 68 and hsp 89 is evident with longer heat-shocks but this is not as

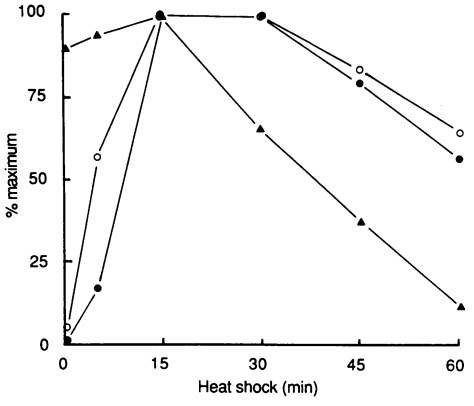

Fig. 2: Effect of increasing duration of heat-shock at 40°C on rates of synthesis of hsp 68, hsp 89 and non-hsp's in 24h larvae.
Larvae were incubated at 40°C for the times indicated before returning to 28°C and labelling and analysing as described. The fluorograph of the gel was scanned in a densitometer and the peaks corresponding to hsp 68 (●), hsp 89 (○) and the non-hsp's (▲) integrated separately. Points are expressed as a percentage of the maximum value attained by each component.

marked as the inhibition of non-hsp synthesis which is virtually complete after a 60 minute shock at 40°C. This information on the kinetics of induction of hsp 68 and hsp 89 in conjunction with the observed differences between cysts and larvae should allow an assessment of the possible roles of bis (5'- adenosyl) oligophosphates in the control of heat-shock and non-heat-shock protein synthesis to be made.

Variation in intracellular Ap_3A and Ap_4A during development

Before measuring nucleotide levels after heat-shock, it is necessary to know the normal intracellular concentrations characteristic of the developmental stages to be used. These have already been reported for Ap_4A [18] but not for Ap_3A in Artemia. Where detected, Ap_3A is found in concentrations higher than those of Ap_4A e.g. 11-fold higher in Dictyostelium and 3-fold higher in BSC-1 monkey cells [25]. The extremely high concentration of 110 pmol/10^6 cells in quiescent Ehrlich ascites cells compared to the low level of Ap_4A of 0.06 pmol/10^6 cells has led to the suggestion that it may act as an antagonist of Ap_4A in cell growth regulation [25].

Decapsulated cysts stored in brine gradually lose their endogenous Ap_4A in a second-order reaction (k = 5.5 l/mol/sec) [18]. Endogenous Ap_3A is lost in a similar fashion (k = 1.1 l/mol/sec) (data not shown). On re-introduction to seawater, the developing cysts rapidly resynthesise both nucleotides reaching maximum values of 3.8pmol/10^6 cells (Ap_4A) and 7.6 pmol/10^6 cells (Ap_3A) by 12h, the time when the

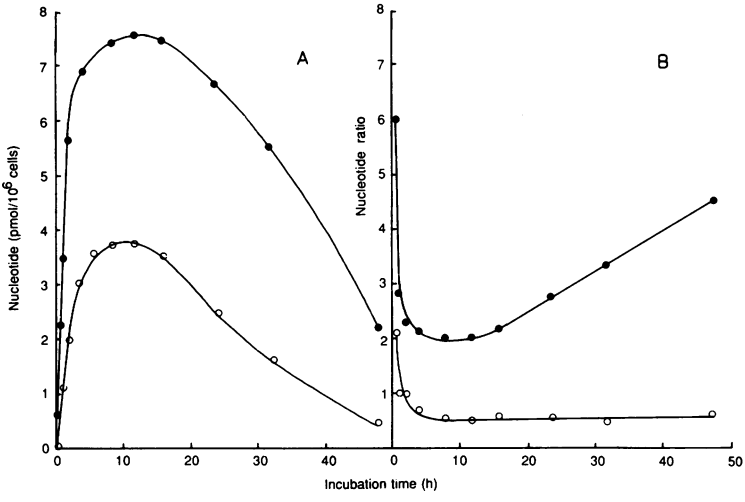

Fig. 3: Variation in intracellular levels of Ap_3A and Ap_4A during Artemia development. (A) Decapsulated cysts which had been stored in brine for 11 days were incubated in seawater for the times indicated. Ap_3A (●) and Ap_4A (○) were extracted and quantitated as described in Materials and Methods. (B) The Ap_3A: Ap_4A ratio (●) was determined for the times indicated using the data from (A). The ADP:ATP ratio (○) at each time was calculated using the data from [18].

cysts begin to emerge and commence DNA replication (Fig. 3A). The exact magnitude of the increases depends on the initial concentrations which in turn depend upon the duration of storage in brine. As shown previously, the level of Ap_4A falls at an average rate of 0.09 pmol/h after hatching, possibly as a result of its consumption as a replicon primer [17,18]. Interestingly, Ap_3A (for which no such role has been proposed) also declines, at an average rate of 0.16 pmol/h. Although the absolute rate of loss of Ap_3A is higher than for Ap_4A, the percentage decrease is less. This is evident from Fig. 3B which shows that, after the initial rapid re-equilibration of nucleotide levels upon rehydration, the ratio of Ap_3A: Ap_4A remains virtually constant at 2:1 during the period of pre-emergence development but increases to 4.5:1 after 48h. This is in contrast to the ADP:ATP ratio which remains unchanged at 1:4 throughout this time. Therefore the levels of Ap_3A and Ap_4A do not simply reflect the concentrations of their respective precursors, ADP and ATP. These findings also suggest that, when seeking a function for these nucleotides it may be important to consider their relative amounts and not just their absolute concentrations.

Effect of heat-shock on Ap_3A and Ap_4A levels in cysts and larvae

When the incubation temperature of 48h larvae is raised from 28° to 32°C no increase in the level of either Ap_3A or Ap_4A is seen (Fig. 4A). On further increasing

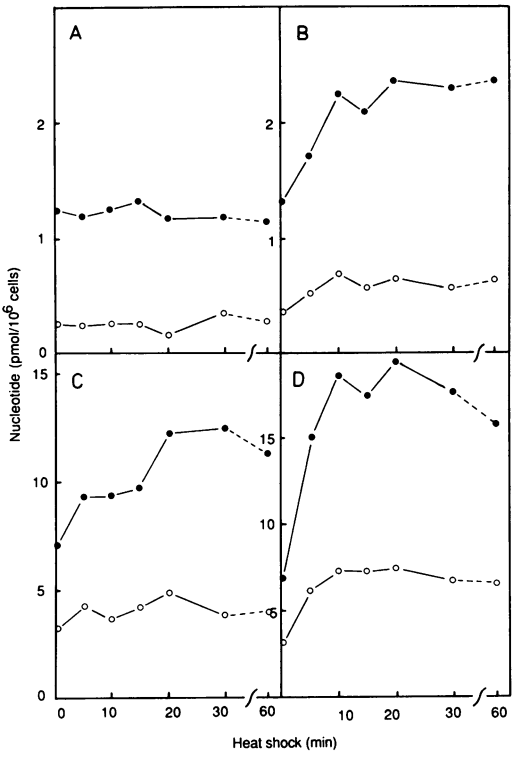

Fig. 4: Effect of heat-shock on intracellular levels of Ap_3A and Ap_4A in cysts and larvae. Cysts and larvae were incubated for various times at the temperatures indicated. Nucleotides were extracted immediately and quantitated as described. (A) 48h larvae, 32°C; (B) 48h larvae, 40°C; (C) 2h cysts, 40°C; (D) 2h cysts, 47°C (●) Ap_3A; (○) Ap_4A.

the temperature to 40°C, however, a slight but reproducible 1.8-fold increase in both nucleotides is evident over a 20 min period though their ratio remains unchanged (Fig. 4B). This increase is similar to that reported for S. typhimurium when it is given a 'mild' heat shock from 28° to 42 °C [8]. When 2h cysts are exposed to a temperature of 40°C they too respond with a 1.7-fold increase in both nucleotides although their initial concentrations are 5 to 10-fold higher (Fig. 4C). As cysts are able to withstand much higher temperatures than larvae and as a heat shock at 50°C causes 10-fold increases in Ap_3A and Ap_4A in S. typhimurium [9], the effect of a heat shock at 47°C on cysts was examined. As expected, greater increases in both nucleotides were found, 2.8-fold for Ap_3A and 2.5-fold for Ap_4A (Fig. 4D) although these were not as great as in S. typhimurium.

DISCUSSION

Increasing the incubation temperature of Artemia cysts and larvae causes an increase in the rates of synthesis of Ap_3A and Ap_4A relative to their rates of degradation and this results in new and higher steady-state levels of these nucleotides. The question is - does this have any physiological significance, particularly with relation to the heat-shock response?

We feel that our results do not support a direct relationship between the temperature-dependent increases in Ap_3A and Ap_4A and the increased synthesis of heat-shock proteins for the following reasons:

(1) the synthesis of hsp 89 is increased at 32°C, yet no increase in either nucleotide occurs during incubation at this temperature (Figs 1A and 4A).

(2) the synthesis of hsp 70 and especially hsp 89 is substantially increased in larvae by a 5 min heat-shock at 40°C yet this treatment causes only a slight 1.3-fold increase in the levels of Ap_3A and Ap_4A (Figs. 2 and 4B). It is difficult to imagine how this small change or even the 1.8-fold increase seen after 20 min could cause the dramatic alterations to the patterns of protein synthesis that are observed.

(3) the absolute concentrations of Ap_3A and Ap_4A in non-heat-shocked 12h cysts are 3- and 6-fold higher respectively than the highest levels reached in heat-shocked 48h larvae yet this does not lead to the induction of heat-shock proteins in the cysts (Figs. 1C, 3 and 4B); the temperature also has to be increased.

Similarly, we can find no evidence to suggest that enhanced levels of these nucleotides are responsible for the repression of non-heat-shock protein synthesis since:

(1) their absolute concentrations are higher in non-heat-shocked 2h and 12h cysts, which maintain non-hsp synthesis at 40°C, than in heat-shocked 48h larvae which repress the synthesis of proteins other than hsp68 and hsp89 after 1h at this temperature (Figs. 1, 3, and 4B).

(2) the relative increases in these nucleotides are identical in cysts and larvae which are heat-shocked at 40°C yet the larvae repress non-hsp synthesis while the cysts do not (Figs. 4B and C).

(3) exposure of 2h cysts to a temperature of 47°C results in the highest levels of Ap_3A and Ap_4A yet to be detected in Artemia (19.5 and 7.5 pmol/10^6 cells respectively) yet even these levels do not cause any preferential inhibition of non-hsp synthesis (Figs. 1B and 4D). While it is possible that the gradual reduction in total protein synthesis seen at 45° and 47°C may be due in part to the diversion of the activity of the aminoacyl-tRNA synthetases away from the aminoacylation of tRNA to the production of bis (5'-nucleosidyl) oligophosphates, there is no discrimination between hsp's and non-hsp's.

Finally any slight increases in the ratio of Ap_3A: Ap_4A which were observed during these experiments were also less than those seen during normal larval development. They are therefore unlikely to account for any changes in the transcription or translation of hsp's or non-hsp's. Although they do not appear to induce the heat-shock response, it is still possible that Ap_3A and Ap_4A are involved, possibly as ligands of one or more of the hsp's or non-hsp's, thereby assisting or modifying their function. In this respect we have found that the major Ap_4A-binding protein in Artemia is a protein kinase [28,29], although its in vivo substrate has yet to be identified. Phosphorylation of hsp's could play a major role in their function.

While this manuscript was in preparation, two reports appeared which come to similar conclusions about the relationship between the levels of bis (5'-nucleosidyl) oligophosphates and the heat-shock response. Brevet et al. showed that raising the incubation temperature of cultured Drosophila cells from 19° to 37°C for 1h caused rises of 2.2-fold, 3-fold and 3.3-fold respectively in the levels of Ap_4A, Ap_3A and Ap_3G [30]. The rates of increase of these nucleotides paralleled the known rates of appearance of the Drosophila hsp's. Although high levels of Cd^{2+} (5mM) caused the much more dramatic increases of 100 to 200-fold also seen with this metal ion and S. typhimurium [9], 50μM Cd^{2+} caused no increases although this concentration caused full induction of the hsp's. Hence the authors conclude that while these nucleotides are enhanced by heat-shock and some other stresses, 'they cannot account for all stress-induced activity'. Similarly, Guedon et al. have found that a severe heat shock at 45°C is necessary to increase the level of Ap_4A in Xenopus oocytes, yet hsp synthesis occurs at much lower temperatures, e.g. 33°C [31]. Since microinjected Ap_4A partially represses the heat-induced synthesis of hsp's other than hsp 70, it is suggested that this nucleotide may be responsible for the specific repression of certain hsp's at the end of the stress response. Until more data are available on the endogenous nucleotide levels under such conditions, it is difficult to say how relevant this result is to the normal situation.

In conclusion there appears to be no strong evidence that heat-induced increases in the levels of bis (5'-nucleosidyl) oligophosphates are by themselves responsible for the regulation of the heat-shock response. Their roles as component parts of this response, if any, remain to be established.

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